THE ACTION OF LOW- AND HIGH-CHLORINATED BIPHENYL MIXTURE ON PREPUBERTAL PORCINE OVARY: STEROID SECRETION AND CELLS APOPTOSIS

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Objective. To compare the effects on ovarian function of low chlorinated polychlorinated biphenyls (PCBs) mixture generally distributed at a faster rate and metabolised to a greater extent (Delor 103) with high chlorinated PCBs mixture which are accumulated in large amounts in biological samples (Delor 106).

Methods Theca interna and granulosa cells were isolated from prepubertal porcine follicles and initially cultured in M199/CS without test compounds for 24 hrs to allow for cell attachment to the wells. After 24 h, the medium was discarded and 0.3 ml of fresh M199 alone was added to the control culture, while Delor 103 or Delor 106 at concentrations of 0.02, 0.2 or 2 ng/ml medium were added to the cultures. For analysis of steroid levels, the media were collected after 24 hrs (single exposure) or 72 hrs (repeated exposure) and frozen at – 20 ^{0}C. Additionally, after 72 hrs, LDL test was used to show the action of both mixtures on the cell viability and cells were used for the measurement of caspase-3 activity.

Results. An increase in testosterone secretion after the single exposure and strong stimulatory action on estradiol secretion was noted after permanent treatment with Delor 103. In Delor 106 treated cultures, antiestrogenic action after the single exposure and strong estrogenic action in permanently treated cells was observed. Both mixtures had no effect on cell viability and apoptosis.

Conclusions. By stimulation of estradiol secretion from ovarian follicles in prepubertal animals, PCB mixture could be responsible for premature development of follicles and in consequence, premature sexual maturation.

Key Words: Delor-103 - Delor-106 - Prepubertal ovary - Steroid secretion - Cell viability - Cell apoptosis

Some organic pollutants, including pesticides and some plasticizers, can disrupt normal sexual development in wildlife. The growing number of estrogenic compounds in the environment has also been thought to contribute to early puberty in girls. The normal average age of onset of sexual development in humans is between 12 and 13 years of age. It is not always possible to directly and quantitatively predict the occurrence of substance-induced effects from animal data. *In vitro* systems are uniquely suited to investigations of specific cellular and molecular mechanisms in the ovary and thus could contribute to the risk assessment. Numerous *in vitro* model systems have been described in the literature. Most of the authors used luteinized granulosa cells collected either from superovulated rats or women undergoing *in vitro* fertilization, i.e., not from the ovaries under normal physiological conditions.

In our previously published paper, it has been shown that ortho-substituted congener PCB 153, which is known to have estrogenic properties, accumulated pre-

ferentially in the follicular wall. Concurrently with its accumulation during the first 3 days of exposure, there was a decrease in estradiol secretion by small and medium follicles and no effect on estradiol secretion by large preovulatory follicles while permanent exposure caused huge estrogenic action. Moreover, we showed that the observed changes in estradiol secretion were concomitant with opposite changes in testosterone secretion thus suggesting the modulation of aromatase activity.

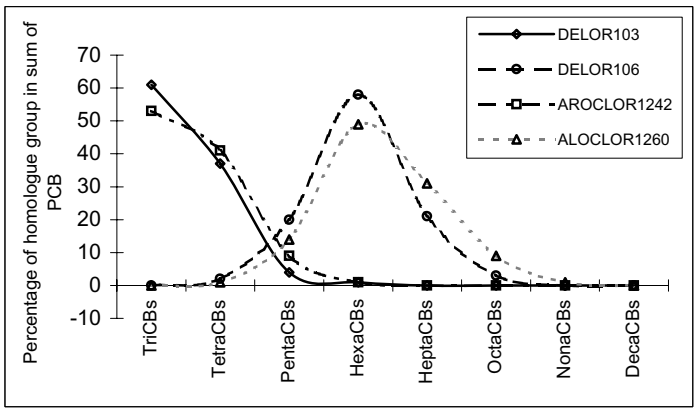


Fig. 1 Proportions of homologues in former commercial PCB mixtures.

However, organisms in the environment are not only exposed to a single substance but they are subjected to the simultaneous action of a multitude of chemicals with endocrine activity. It has previously been shown that the effects of a combination of estrogens in several *in vitro* tests can be predicted by addition of their concentration, even when the individual effects are insignificant. A number of substances are known to be estrogen receptor agonists, belonging to different classes of chemicals, such as estrogenic steroids (natural and synthetic), phyto- and mycoestrogens, and xenoestrogens (e.g. pesticides, alkylphenols, plasticizers). Several xenoestrogens exhibit not only the estrogenic activity, but also cytotoxicity. Some commercial products (under major trade names such as: Aroclor, Kanechlor, Clophen, and others) are all mixtures of several PCB congeners (209 are theoretically possible) and have been sold as different grades, based on their chlorine content.

Taking into the consideration that patterns of biological action dependent on the structures the aim of the present study was to compare dose- and time-dependent effects of the low-chlorinated (mostly trichlorinated) PCB mixture (Delor 103) and high-chlorinated (mostly hexachlorinated) PCB mixture (Delor 106) formerly produced in the Czech Republic. In general, highly chlorinated PCBs are poorly metabolized and thus their elimination is slow (Sipes and Schnellmann 1987). On the other hand, low-chlorinated PCBs are hydroxylated in vitro and in vivo (McLean et al. 1996; James, 2001). The effects of technical polychlorinated biphenyl (PCB) preparations - Delors 103, 104, 105, and 106 – which were produced formerly in Czechoslovakia, were tested in this study. The congener patterns of Delors 103 and 106 resembled these of Aroclors 1242 and 1260, respectively (Fig. 1).

In our laboratory, we collect follicular cells from porcine ovary excised from animals showing natural estrous cycle to study the direct effects of various xenobiotics on ovarian steroidogenesis. We use a co-culture model of theca and granulosa cells cultured at the ratio existing in vivo in the follicles. This model is more physiological than the culture of individual cell types or even cell lines. In this co-culture model, both cell types retain their follicular stage-specific ability to secrete steroids (estradiol, testosterone and progesterone) and thus they are ideally suited to investigation of the effects of xenobiotics on hormone production. We have employed this model previously for investigating the action of gonadotropins on ovarian cell function (Stoklosowa et al. 1982; Gregoraszczuk et al. 1988; Gregoraszczuk and Skalka 1996), for testing of direct action of antiepileptic drugs on the ovarian steroidogenesis (Tauboll et al. 2002; Tauboll et al. 2003) and for examination of dose- and time-dependent action of different xenobiotics, like PCBs on steroid secretion by follicular cells (Wojtowicz et al. 2000; Gregoraszczuk et al. 2003). In our previous experiments, we investigated the action of a single congener (PCB 153 and PCB 126) on follicular steroidogenesis.

Materials and Methods

Chemicals and reagents Parker medium M199, trypsin, and calf serum (CS) were purchased from the Laboratory of Sera and Vaccines, Lublin Poland. Antibiotic Antimycotic Solution, stabilized (100x) (Penicilin 20units/ml, Streptomycin 0,02mg/ml, Amphotericin B 0,05ug/ml) was obtained from Sigma Chemical Co. St. Louis, MO, USA. DELOR 103 or DELOR 106 mixtures (prepared from standard solutions by di-

lution with EtOH) were purchased from Slovak Metrological Institute. A comparison of the homologue compositions of these mixtures with that of the more well-known Aroclors is presented in Figure 1.

Chemical analysis. A mixture of $^{13}C_{12}$ labeled PCB (mono to deca chloroCB) was added to each sample prior extraction. Medium samples were extracted three times with 20, 10 and 10 ml of hexane. Tissue samples were homogenized with anhydrous natrium sulphate and extracted with hexane/acetone (1/1 v/v) in a Soxhlet exctractor for six hours. The extracts were cleaned on combined silica column (activated silica gel, H_2SO_4 deactivated silica gel and NaOH deactivated silica gel). Eluates were concentrated to 100 µl in heptane and analysed by GC/MS/MS. The GC/MS/MS method was validated using certified reference materials NIST 1944 and 1588. Standard mixtures of Delor 103 and 106 and Arochlor 1242 and 1260 with added internal standards were analyzed by the same method.

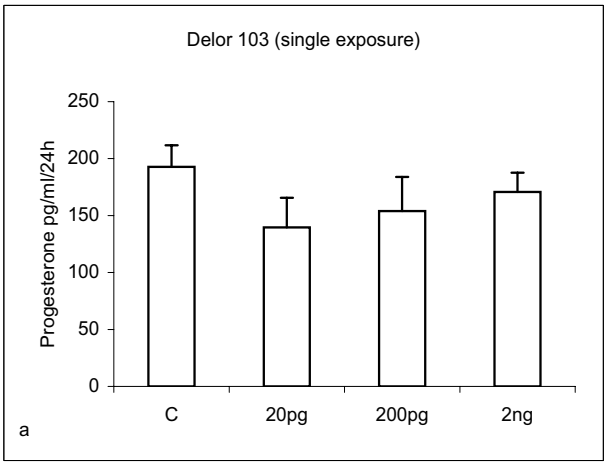
Cell cultures. Ovaries from prepubertal pigs were collected at a local abattoir. Approximately 1.5 h elapsed from slaughter to collection in the laboratory. Granulosa cells (Gc) and theca interna cells (Tc) were isolated from the follicles (4-6 mm in diameter) and prepared according to the technique described by Stoklosowa et at (1978). Gc were scrubbed from the follicular wall with round-tipped ophthalmologic tweezers and rinsed several times with PBS. After isolation, Gc were washed three times in M199, collected and suspended in M199 supplemented with 5% CS (M199/CS). The Tc from the same follicles were prepared as previously described in detail by Stoklosowa et al.(1978). Briefly, the theca layers were placed in a drop of saline under the dissecting microscope. The theca interna was separated manually from the underlying theca externa. The isolated theca interna tissue was then washed with PBS, cleaned, cut with scissors and exposed to trypsinization with 6-7 ml of 0.25% trypsin in PBS for 10 min at 37° C. The cells were separated by decantation and the procedure was repeated three times. Finally, the cells were centrifuged and resuspended in M199/CS. For co-culture experiments, viable granulosa and theca cells, as determined by the Trypan blue exclusion test, were inoculated at a concentration of 3.6 x 10^{-4} Gc/well and 0.9 x 10^{-4} Tc/well in Nunc 96 well tissue culture plates. Thus, the concentrations were comparable to that observed in vivo (Gc: Tc = 4:1) according to Stoklosowa et al. (1982). The cultures were maintained at 37° C in a humidified atmosphere of 5% CO_2/95% O_2.

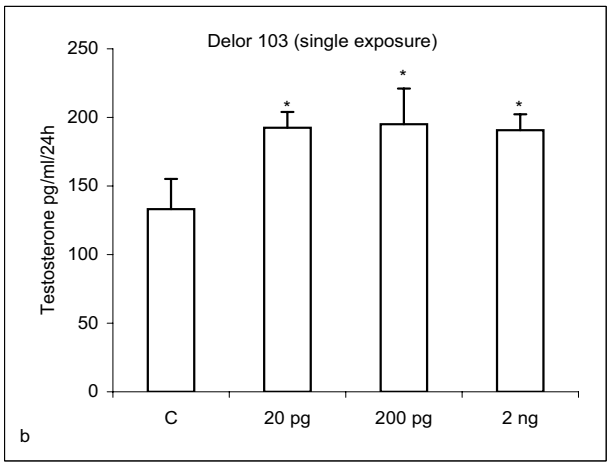
Experimental procedures. *Experiment 1* was conducted to examine the concentration- and time-dependent effects of Delor 103 and Delor 106 on testosterone (T) and estradiol (E2) secretion by follicular cells. The cells were isolated and initially cultured in M199/CS without test compounds for 24 h to allow for cell attachment to the wells. After 24 h, the medium was discarded and 0.3 ml of fresh M199 alone was added to the control culture, while Delor 103 or Delor 106 at concentrations of 0.02, 0.2 or 2 ng/ml medium were added to the experimental cultures. Media were changed every 24 hr, and a new portion with an appropriate dose of Delors were added to the experimental groups. For analysis of steroid levels, the media were collected after 24 h (single exposure) or 72 h (repeated exposure) and frozen at – 20 ^{0} C. After each collection, fresh medium without or with 0.02, 0.2 or 2 ng/ml of the appropriate test compound was added to the cultures. Every treatment was conducted in 4 wells and each experiment was repeated 3 times.

Stock solutions of these test compounds in EtOH were prepared and added to M199 supplemented with 5% CS. The final concentration of EtOH in the medium was in each case 0.5% EtOH at the concentration used in these experiments, had no effect on steroid secretion and cell viability (date not show).

Experiment 2. Taking into account data of Rogers et al (1983) who suggested that Aroclor 1016 affected membrane integrity and the associated metabolic functions in CHO-K1 cells, this experiment was conducted to examine the concentration-dependent effects of Delor 103 and Delor 106 on cells viability. LDL test was used to show the action of both mixtures on cell viability. The media were collected after 72 hrs of culture. This time of exposure was chosen taking into account our previous study which showed that 24-hr culture with all investigated by us compounds (TCDD, PCB 126, PCB 153, PCB3 or DDT) had no effect on cell viability (Augustowska and Gregoraszczuk 2004; Gregoraszczuk and Wojtowicz 2002; Ptak et al. 2004), while in the same experimental design, longer time of exposure caused a decrease in cell viability (Ptak et al. 2004). Every treatment was conducted in 4 wells and each experiment was repeated 3 times.

Experiment 3. Because over 99 % of ovarian follicles undergo atresia, any chemical that hastens this process could have potentially devastating effects on fertility. For this reason, the present experiment was conducted to examine the concentration-dependent effects of Delor 103 and Delor 106 on cells apoptosis. At the





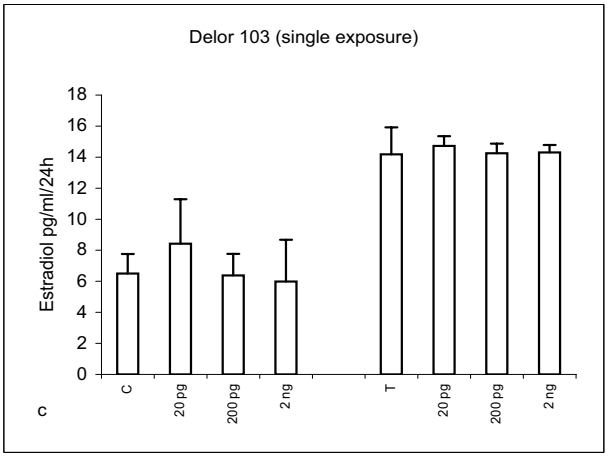


Fig. 2 The effects of single exposure to Delor 103 on a) basal progesterone, b) basal testosterone and c) basal and testosterone (T) stimulated estradiol secretion by co-culture of the theca and granulosa cells isolated from ovarian follicles collected from prepubertal animals. * p<0.05

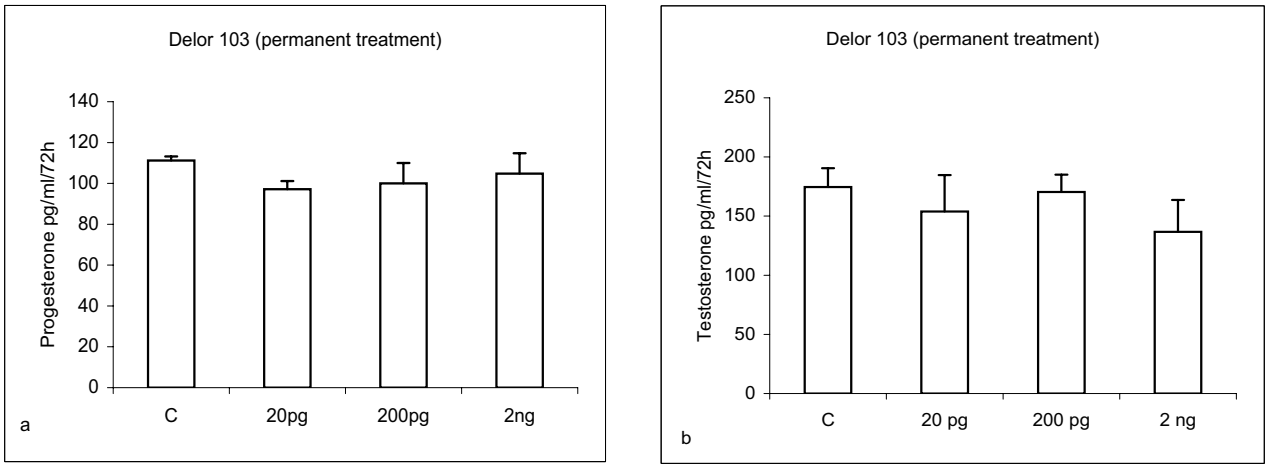
end of culture, the cells were used for the measurement of caspase-3 activity to show the action of both mixtures on cells apoptosis.

Cell viability. *Lactate dehydrogenase cytotoxicity assay:* Cytotoxicity detection kit (Roche Applied Science) is colorimetric assay for quantification of cell death and cell lysis based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into supernatant. An increase in the amount of dead or plasma membrane-damaged cells results in an increase of the LDH activity in the culture supernatant. After 72-hour treatment of the cells with increasing doses of Delor 103 and Delor 106, the culture supernatants were collected and incubated with the reaction mixture from the kits. After 30 min, the reaction was stopped by adding 1N HCl and absorbance was measured. The absorbance was measured at a wavelength of 490 nm with a reference wavelength of 600 nm in microELISA plate reader.

Caspase-3 activity. The cultured cells were lysed with a lysis buffer containing 50 nm Hepes, pH 7.4, 100 nM NaCl, 0.1% CHAPS, 1 nM EDTA, 10% glycerol, 10 nM DTT. The soluble fraction of the cell lysate was then assayed for caspase-3 activity using AcDEVD-Pna (Sigma), a colorimetric substrate for caspase-3 , as described in the manufacturer's protocol.

Steroid analysis. E2 (estradiol), P (progesterone), T (testosterone) were determined radioimmunologically using Spectra kits (Orion, Diagnica, Finland), supplied by Polatom (Swierk, Poland). Assay sensitive for E2, P and T were 5 pg/ml, 94 pg/ml and 5 pg/ml, respectively. Inter- and intra-assay variation coefficients for estradiol were 10.28 % and 2.9 %, respectively. Inter- and intra-assay variation coefficients for progesterone were 5.8 % and 2.9 %, respectively. Inter- and intra-assay variation coefficients for testosterone were 5.4 % and 5.3 %, respectively.

Statistical evaluation. Each treatment was repeated three times in quadruplicates and thus the total number of replicates was 12. Since the variations between the experiments were small, those 12 results were averaged. Statistical analysis was performed using SAS System 9.0. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparison procedure. Log concentrations were used for all calculations. Multiple way (three-way and two-way) analyses of variance were tried, but significant interactions were found, prohibiting this type of



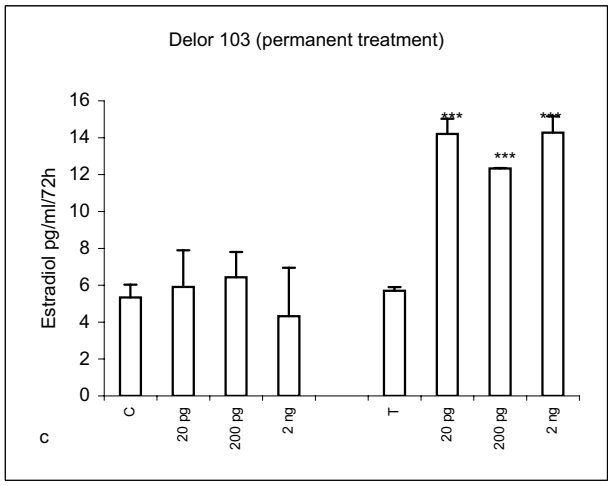


Fig. 3 The effects of permanent treatment with DELOR 103 on a) progesterone, b) testosterone and c) estradiol secretion by co-culture of the theca and granulosa cells isolated from ovarian follicles collected from prepubertal animals . *** p<0.001

analysis. The data in the figures are presented as percent of corresponding control with each average (n = 12) expressed as the mean ± SEM.

Results

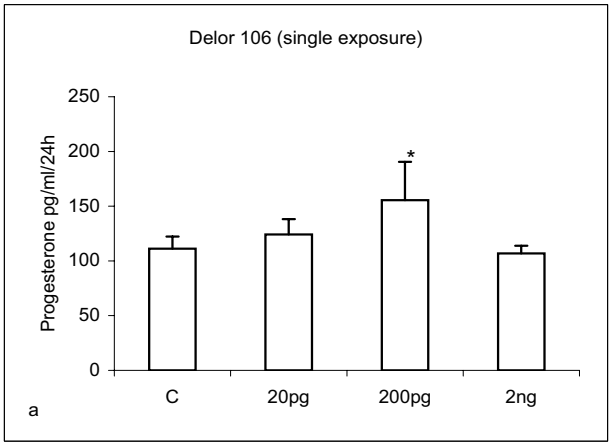
Single and permanent treatment with Delor 103. A single exposure to Delor 103 caused no effect on progesterone secretion with parallel increase in testosterone secretion (192.5, 195.0 and 190.65pg/ml/24h after 20pg, 200pg and 2ng/ml respectively vs. 133.13pg/ml/24h in control; p<0.05; Fig.2a,b). No effect on estradiol secretion was observed in both control and testosterone-supplemented cultures.(Fig.2c).

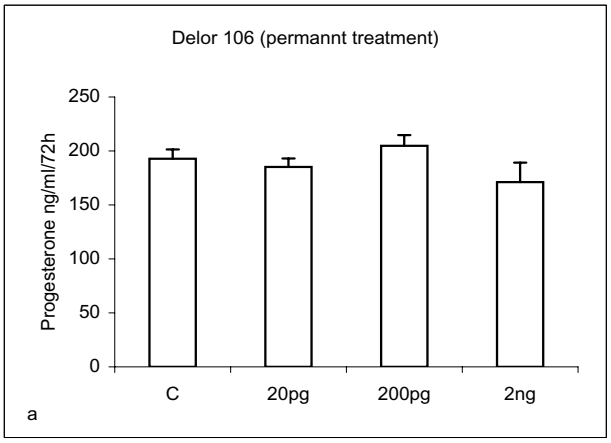
Permanent treatment was without effect on progesterone (Fig.3a) and testosterone (Fig.3b) secretion. Strong stimulatory action on estradiol secretion was noted in cultures supplemented with testosterone as a substrate for estradiol secretion (14.21, 12.34 and 14.28pg/ml/72h after 20pg, 200pg and 2ng/ml respectively vs. 7.5pg/ml/72h in control; p<0.001; Fig.3c).

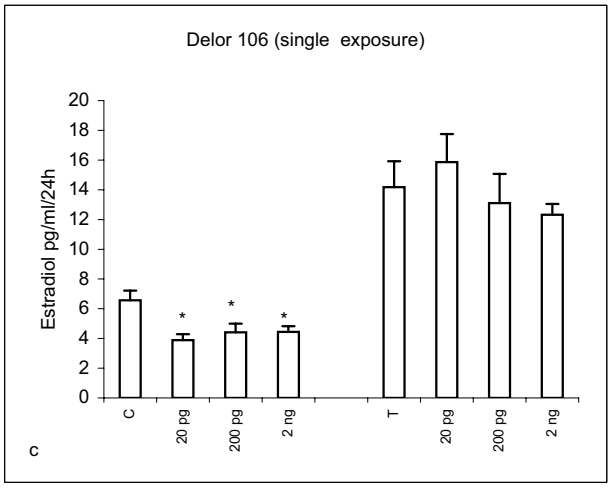
Single and permanent treatment with Delor 106. Delor 106 at a dose of 200 pg/ml increased progesterone (155 pg/ml/24h vs. 111.1 pg/ml/24h in control) and testosterone secretion (186.5 pg/ml/24h vs. 133.13pg/ml/24h in control; p<0.05; Fig. 4a,b). The decrease in estradiol secretion under the influence of all used doses of the preparation (3.8pg, 4.4pg and 4.4pg/ml/24h vs. 6.56 pg/ml/24h in control; p<0.05) was noted under basal conditions but not in cell treated with testosterone (Fig. 4c).

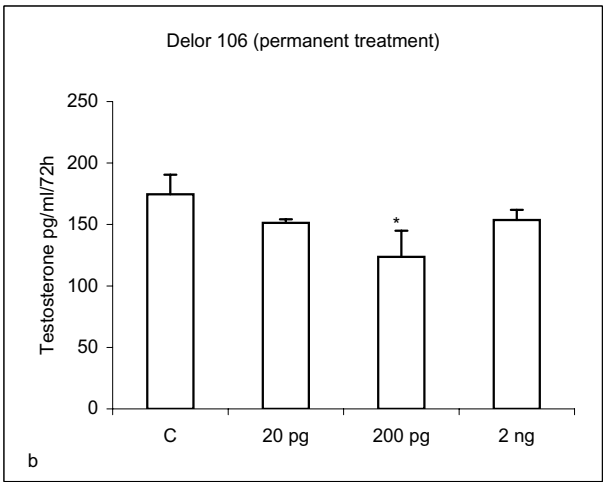
Permanent treatment was without effect on progesterone release (Fig. 5a). The dose of 200 pg/ml decreased testosterone secretion (123 pg/ml/72h vs. 174 pg/ml/72h) (Fig. 5b). Strong stimulatory action on estradiol secretion was noted in cultures supplemented with 20 pg and 200 pg/ml (8.0 pg and 7.59 pg/ml/72h vs. 5.06 pg/ml; p<0.05) under basal conditions and under the influence of all doses of the preparation in testosterone-supplemented cultures (14.17 pg, 12.44 and 12.88 pg/ml/72h after 20 pg, 200 pg and 2 ng/ml, respectively, vs. 4.88 pg/ml/72h in control; p<0.001; Fig. 5c).

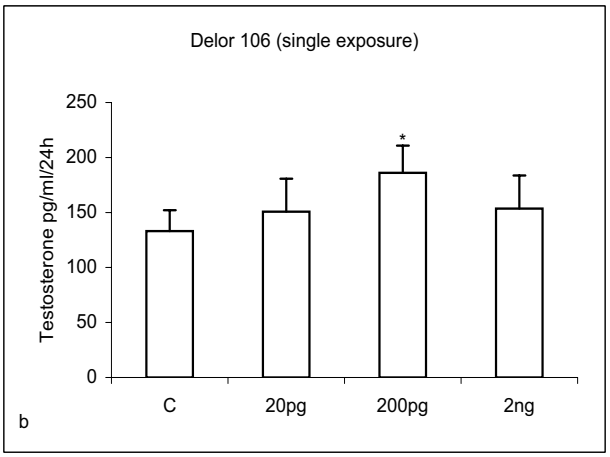
Action of Delor 103 and Delor 106 on cell viability and apoptosis. Both mixtures had no effect on cell viability and apoptosis (Fig.6a,b).











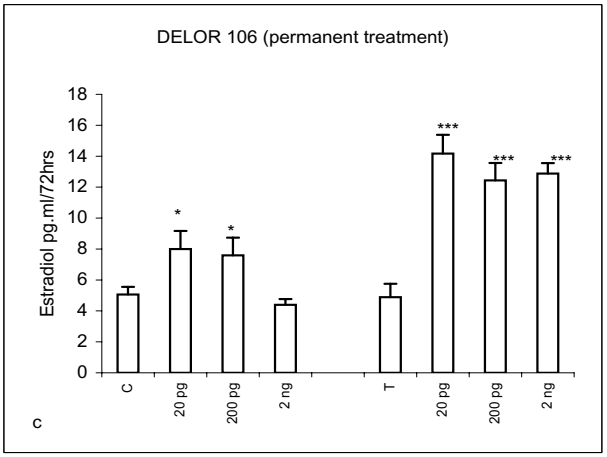


Fig. 4 The effects of single exposure to Delor 106 on a) progesterone, b) testosterone and c) estradiol secretion by co-culture of the theca and granulosa cells isolated from ovarian follicles collected from prepubertal animals. * p<0.05

Fig. 5 The effects of permanent treatment with Delor 106 on a) basal progesterone, b) basal testosterone and c)basal and testosterone (T) stimulated estradiol secretion by co-culture of the theca and granulosa cells isolated from ovarian follicles collected from prepubertal animals.
*** p< 0.05. *** p<0.001**

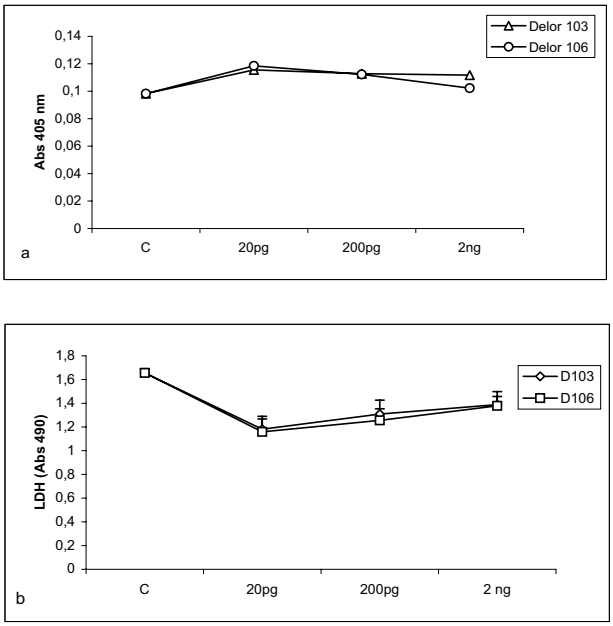


Fig. 6 The effect of Delor 103 and Delor 106 on a) cells apoptosis and b) viability.

Discussion

The results of the presented study showed that the low-chlorinated mixture Delor 103 first showed androgenic action and then after longer exposure, it stimulated P450 arom activity and strong estrogenic action was clearly visible. Certain PCB congeners, putative PCB metabolites and complex PCB mixtures have been reported to be estrogenic (McKinney and Waller 1994). The estrogenic effects of the organochlorine compounds are much less potent (at least 2 orders of magnitude) than those of 17β-estradiol. However, the organochlorine compounds with long biological half-lives accumulate in the food chain and might reach concentrations high enough to elicit significant estrogenic effects

Stimulatory action of PCBs on testosterone secretion, which is essential for initiation of follicular development and then activates P450arom, participating in conversion of testosterone to estradiol, could be responsible for premature development of follicles. In consequence, follicles can reach faster the stage of the preovulatory follicles. Taking into consideration that during the early stage of follicular differentiation, androgen acts as an enhancer of FSH-stimulated follicular differentiation, stimulatory action of the mixtures on testosterone secretion by cells collected from prepubertal animals can explain observations of earlier maturation after exposure to PCB mixture (Teilmann et al. 2002). A recent study has shown that young girls in the U.S. appear to be developing signs of puberty at a younger age than previously believed. Sporadic cases of precocious puberty in girls have been linked to DES or estrogens in personal care products. Early menarche in girls has been linked to prenatal/lactational exposure to polybrominated biphenyls (Blanck et al. 2000) and premature breast development was associated with their serum phthalate levels (Colon et al. 2000).

Krstevska-Konstantinova et al. (2001) suggested that precocious puberty and DDT/DDE exposure might be related. To our knowledge, there are only two reports on age at menarche: one suggested that earlier menarcheal age was associated with PBB exposure, while the other found no difference in relation to PCB exposure (Blanck et al. 2000; Den Hond et al. 2002). However, a different endocrine activity may be ascribed to different halogenated compounds. Menarche is linked to the elevated estrogen production (Apter 1980; Apter and Vihko, 1985; Legro et al. 2000). The PCB mixture tested by us may exert such an estrogenic effect. Thus, we hypothesize that neonatal exposure to the estrogenic effect of PCBs may result in precocious puberty.

Different observations have been made in Delor 106 treated cells. A single exposure to this preparation caused antiestrogenic action while long-term exposure led to estrogenic action suggesting biphasic action of high-chlorinated biphenyls. Although most of the antiestrogenic actions of PCBs can be explained by effects on estrogen metabolism, there is also a clear possibility of their action at estrogen receptors. Furthermore, the effects of PCB metabolites may be different from that of the parent compound. For example, Pang and co-workers (1999) found that 3,4,5-TrCB was a potent inducer of CYP1A1 and CYP1B1 mRNA and a promoter of estrogen metabolism by both 2- and 4-hydroxylation.

The "hormonal activity" described for PCBs has been frequently attributed to estrogen-enhancing (estrogenic) or estrogen-blocking (anti-estrogenic) activity. However, "endocrine disruption" is not a toxic endpoint per se, but may be used to describe a mechanism through which toxicity may occur and should be used in this capacity. The presented study investigated a relationship between time of exposure and cell viability. Surprisingly, both Delor 103 and 106 at all used doses had no effect on cell viability. The complexity of PCB mix-

tures and their thermal breakdown products have made this group of chemicals difficult to characterize in terms of toxicity. It is important to recognize that toxicity of PCB mixtures is dependent on the toxicity of the individual PCB congeners present within the mixture, and of course, on the presence of other more potent contaminants (SAFE 1994). Some PCBs and their thermal degradation products are much more toxic than others. Moreover, thermally or environmentally degraded PCB mixtures significantly deviate in composition and consequent toxicity from the original commercial PCB mixture (MCFARLAND and CLARKE 1989; SAFE 1990; BORLAKOGLU at al. 1990). In addition, studies conducted to assess human health risks have been complicated by the presence of other, known toxic agents, such as methyl mercury, pesticides, etc. Therefore, the careful examination of specific contaminant mixtures is evolving as an important tool in gauging health risk (SAFE 1994)

In conclusion, the stimulatory action of PCBs mixture on estradiol secretion with no effect on follicular cell viability and apoptosis, observed in the presented study, could be one of many mechanisms engaged in the action of these compounds as factors advancing sexual maturation. Changes in the age of menarche as the result of environmental, nutritional, or pathophysiological factors (such as in women with central precocious puberty) allow an earlier onset of cyclic follicle recruitment.

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